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Parathyroid hormone-related protein protects against kainic acid excitotoxicity in rat cerebellar granule cells by regulating L-type channel calcium flux

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Abstract

The parathyroid hormone-related peptide (PTHrP) and PTH/PTHrP receptor genes are widely expressed in the CNS and both are highly expressed in the cerebellar granule cell. We have shown previously that PTHrP gene expression in granule cells is depolarization-dependent in vitro and is regulated specifically by Ca^{2+} influx via L-type voltage-sensitive calcium channels (L-VSCCs). Kainic acid induces long-latency excitotoxicity in granule cells via L-VSCC-mediated Ca^{2+} influx. Here, we show that PTHrP is just as effective as the L-VSCC blocker, nitrendipine (NTR), in preventing kainate excitotoxicity. A competitive inhibitor of PTHrP binding abrogates its neuroprotective effect. Both NTR and PTHrP decrease $^{45}Ca^{2+}$ influx to the same degree. These findings suggest that PTHrP functions in an autocrine/paracrine neuroprotective feedback loop that can combat L-VSCC-mediated excitotoxcity.

Keywords: Cerebellar granule cell; L-type calcium channel; Excitotoxcity; Neuroprotection; Parathyroid hormone-related protein; Kainic acid

Parathyroid hormone-related protein (PTHrP) was discovered in the late 1980s as the tumor product that is responsible for most instances of malignancy-associated hypercalcemia [12]. PTHrP and parathyroid hormone (PTH) are now known to be members of a small gene family. One result of this common heritage is a stretch of highly homologous sequence at the N-terminus of each peptide and another is the curious fact that these N-terminal products appear to be serviced primarily by a single G protein-coupled receptor, referred to as the type 1 PTH/PTHrP receptor [12]. This is unusual because the functions of PTH and PTHrP are so remarkably different. PTH is a classical peptide hormone charged with regulating systemic mineral homeostasis and it does so via type 1 receptors in bone and kidney. PTHrP does not normally circulate and the PTHrP and the type 1 receptor genes are widely expressed in adult and fetal tissues, often in a hand-in-glove fashion that bespeaks paracrine function [12]. In embryonic and neonatal life, PTHrP serves as a developmental regulatory factor that regulates processes as varied as the development of endochondral bone and the mammary epithelium and the eruption of teeth [8,13,20]. The best defined PTHrP function in the adult is as a stretch-induced smooth muscle relaxant that allows structures such as the bladder, stomach and uterus to accommodate gradual filling [3,9].

Something is known of the mechanisms by which PTHrP carries out these functions. In several of its developmental roles, PTHrP acts by regulating the rate at which programs of differentiation proceed [8,12]. The terminally differentiated cell in these programs is an apoptotic cell, so that by retarding the progress of the program in question PTHrP decreases apoptosis; one attractive proposal is that PTHrP might do so by upregulating Bcl-2 [1,12]. In smooth muscle, PTHrP appears to produce relaxation by inhibiting Ca^{2+} influx via voltage-gated dihydropyridine-sensitive calcium channels (L-VSCCs) [11,12].

The PTHrP and type 1 receptor genes are also widely expressed in the CNS, most particularly by neurons that contain a high density of L-VSCCs [17,18]. The cerebellar granule cell is one such neuron and granule cells both produce and respond to PTHrP [6]. In vitro, PTHrP mRNA expression in granule cells is depolarization-depen-

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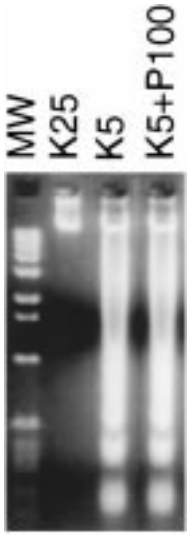

Fig. 1. DNA laddering in depolarized and repolarized granule cells. Cells were maintained in a depolarized state in 25 mM K and were repolarized in 5 mM K, alone or with PTHrP at 100 nM. MW, markers; K25, 25 mM K; K5, 5 mM K; K5 + P100, 5 mM K + 100 nM PTHrP.

dent and is triggered specifically by L-VSCC-mediated Ca^{2+} influx and not by Ca^{2+} entry by other routes [6]. Cerebellar granule cells also have an interesting developmental history in that their survival in vivo is depolarization-dependent and is achieved by establishing contact-induced depolarization by mossy fibers; granule cell precursors that do not establish such connections succumb to a classic apoptotic death [4,6].

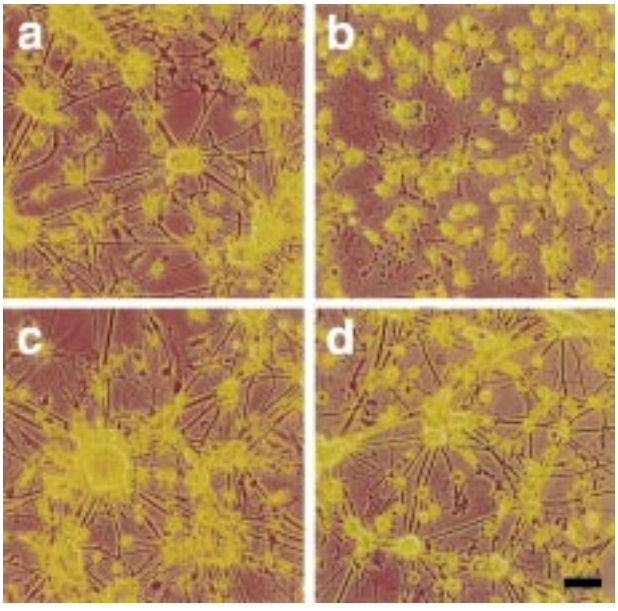

Fig. 2. Granule cell cultures visualized by phase-contrast microscopy. (a,b) Under depolarizing conditions, the cells are healthy (a), but a majority have died after exposure to kainic acid (b). (c,d) Neuroprotection provided by NTR (d) and PTHrP (c). Scale bar, 45 μm.

In work reported here, we pursued two leads. The first was a consideration of whether PTHrP might serve as a brake on the apoptotic fate of granule cells when grown under repolarizing conditions in vitro. The second was an examination of whether PTHrP might regulate L-VSCC function in granule cells as it appears to do in smooth muscle cells and in so doing be neuroprotective in vitro.

Primary cultures of rat cerebellar granule cells were prepared from 8-day-old pups and maintained in 6-well culture plates in complete medium containing 25 mM KCl, as described [6]. All experiments were carried out at 8 days in vitro. Apoptosis was induced by placing the cells in serum-free synthetic medium containing 5 mM K for 24 h and DNA fragmentation was analyzed as described [4]. PTHrP (1–36) amide was measured in conditioned medium using a PTHrP (1–36) IRMA [14]. To study excitotoxcity, medium was removed, rapidly mixed with the agent(s) in question and replaced for 24 h. Nitrendipine (NTR) was used at 100 nM in order to avoid its non-specific effects when used in micromolar concentrations (reviewed in Ref. [6]). PTHrP was used at 100 nM because its half-maximal stimulation in granule cells is at 16 nM [6]. All reagents were from standard suppliers.

Cell death was assessed using phase-contrast microscopy and quantified by propidium iodide staining [7]. We used the automatic grain counting program within MCID (Imaging Research, Ontario, Canada) to determine the fraction of neurons killed under each experimental condition. Specifically, a region to be automatically scanned included only the area corresponding to regions of neuronal aggregates. Within these regions to be scanned, the total area corresponding to the round, brightly florescent nuclei of dead neurons was determined for 3–6 aggregates per low power field. A minimum of three separate fields for each condition was analyzed. The fractional neuronal kill was calculated by the ratio of areas corresponding to propidium-labeled nuclei and the total area of the neuronal aggregate. Repeat determinations showed that this technique provided reproducible numbers within 1–2%. The $^{45}Ca^{2+}$ flux assay was carried out over 10 min as described [6].

As noted, cerebellar granule cells survive when grown under depolarizing conditions (25 mM K) in vitro, mimicking their depolarization-dependent survival in vivo, and undergo apoptosis when repolarized (5 mM k) in vitro [4,6]. This process is prevented by forskolin/cAMP and appears to involve regulation of Bcl-2 levels, so that PTHrP seemed to be a particularly attractive candidate for regulation of granule cell survival. Nevertheless, at 10 and 100 nM, PTHrP (1–36) amide had no effect whatsoever on granule cell survival in 5 mM K, as assessed either by propidium quantitation or by DNA laddering (Fig. 1). This point was confirmed in three separate experiments. In order to assess the possibility that exogenous peptide was being too rapidly degraded to exert such effects, we measured PTHrP by N-terminal IRMA and found that the added

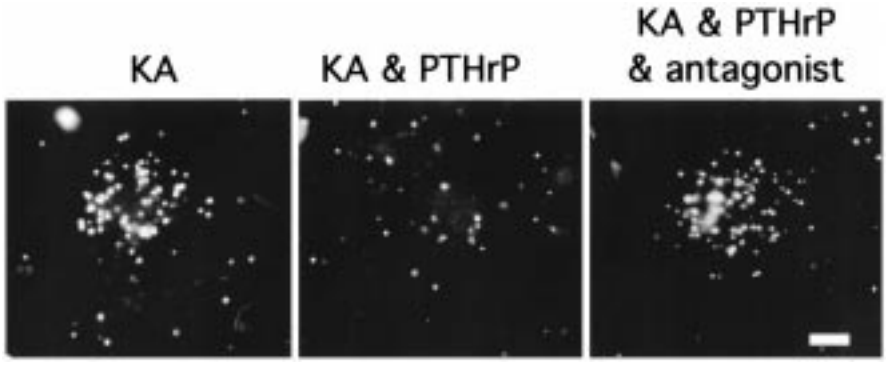

Fig. 3. Cell death assessed by propidium iodide staining. Propidium iodide can bind to nuclear DNA only when the cell membrane is not intact and thus identifies a dead cell; each bright dot represents the nucleus of a dead cell. The left panel shows kainic acid (KA) alone, the middle panel KA plus PTHrP and the right panel KA together with PTHrP and the PTHrP antagonist. The percent kill (±SEM) under these three conditions was 23 ± 3% ($n = 10$), 2 ± 2% ($n = 11$, $P < 0.001$ with respect to KA alone) and 23 ± 2% ($n = 10$), respectively. Scale bar, 25 μM.

peptide appeared to be fully intact at 24 h (IRMA values 90–100 nM).

As is the case for many neurons, granule cells are subject to two forms of excitotoxic death, the nature of which appears to reflect the degree of insult [2,19]. High concentrations of glutamate acting on NMDA receptors cause a generalized influx of cations and a collapse in mitochondral function leading to almost immediate necrosis [2]. Lower concentrations of glutamate, or exposure to other excitotoxins such as kainic acid that act on non-NMDA receptors, trigger Ca^{2+} entry specifically via L-VSCCs and lead to excitotoxcity characterized by a long-latency (6–24 h or so) [2,19]. Given the fact that PTHrP gene expression is regulated in granule cells by L-VSCC activity [6] and N-terminal PTH/PTHrP sequences regulate L-VSCC conductance in other systems [9,11,12], we tested the idea that PTHrP might decrease L-VSCC-mediated Ca^{2+} influx in granule cells and thereby be neuroprotective.

We found that kainic acid at 60 μM induced long-latency excitotoxcity in granule cells, producing approximately 20–50% cell death at 24 h as assessed by phase-contrast microscopy (Fig. 2) and quantitated by propidium staining (Fig. 3). This excitotoxcity was eliminated when the dihydropyridine calcium channel antagonist, NTR, was included at 100 nM, confirming that it is VSCC-mediated. PTHrP (1–36) amide at 100 nM was found to be equally neuroprotective, reducing cell death to background levels (2 ± 2%, SEM) at 24 h (Fig. 3). The stimulatory effects of N-terminal PTH/PTHrP sequences on the type 1 receptor can be completely blocked by a 10-fold molar excess of a competitive inhibitor of peptide binding, [$Nle^{8,18}Tyr^{34}$] bPTH (3–34) amide [12], and we found that coaddition of this inhibitor at 1μM eliminated entirely the neuroprotection provided by PTHrP (Fig. 3). Glutamate (50 μM) induced immediate necrosis and neither NTR nor PTHrP affected this process (not shown).

These findings suggested that, like NTR, PTHrP might be acting to somehow limit L-VSCC-mediated Ca^{2+} flux. We examined this question by studying the effects of PTHrP and NTR on $^{45}Ca^{2+}$ flux associated with depolarization in granule cells. Both agents appeared to produce an equivalent decrease in $^{45}Ca^{2+}$ flux (Fig. 4). These effects were not additive (Fig. 4).

PTHrP regulation and/or effects in the CNS have only recently come under study. Using the granule cell system, we found previously that PTHrP gene transcription was under the control of L-VSCC-mediated Ca^{2+} influx that tracked via the calmodulin-CaM kinase cascade [6]. This control was entirely specific to L-channel Ca^{2+} influx, in that Ca^{2+} entry via other means had no influence whatsoever on the PTHrP gene [6]. This is another example of the specificity of neuronal Ca^{2+} signaling pathways as initially described by Greenberg et al. [5].

The present work is a logical extension of our previous findings in this system as well as published data in smooth muscle systems [9,11,12], which together suggested the

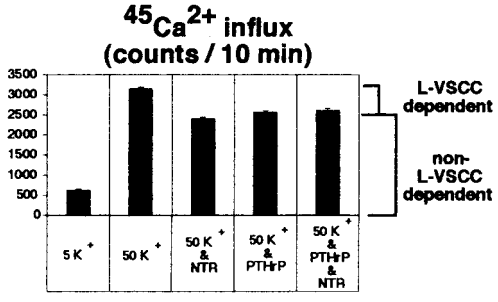

Fig. 4. Depolarization-driven $^{45}Ca^{2+}$ uptake. ^{45}Ca uptake in rat cerebellar granule cells was determined in repolarized cells (5 mM) and 10 min after depolarizing cells with 50 mM K^+ (50 mM) without and with added NTR or PTHrP at 100 nM. Note that depolarization induces Ca^{2+} flux in neurons via a number of channels in addition to the L-VSCC (e.g., the NMDA receptor), so that only a portion of $^{45}Ca^{2+}$ flux is mediated by L-VSCCs (i.e. is dihydropyridine-sensitive at this concentration of NTR). The experiment shown is representative of three separate experiments and the error bars are SEM of six separate wells per condition.

hypothesis that PTHrP might also regulate L-VSCC Ca^{2+} influx in neurons and that this might constitute a neuroprotective feedback loop. In neurons, the sequence appears to constitute an autofeedback loop, in which depolarization-driven L-VSCC Ca^{2+} influx leads to the induction of a gene whose product acts in an autocrine/paracrine fashion to dampen L-VSCC Ca^{2+} influx and thereby provide neuroprotection. At present, we have no insight into the mechanism by which stimulation of the PTH/PTHrP receptor might negatively regulate L-VSCC activity.

We found no evidence that PTHrP had any effect in preventing the apoptotic death of repolarized granule cells. However, Ono et al. [10] have reported that addition of anti-PTHrP (1–34) antiserum prevented granule cell survival when they are cultured under depolarizing conditions, implicating endogenous PTHrP as the product that mediates the depolarization-dependent survival of these cells in vitro [10].

The PTH/PTHrP gene and receptor families are growing and in ways that are relevant to the CNS. At least three PTH-like peptides and two or more distinct receptors are expressed in the CNS [12,15,16], suggesting that the findings described here may presage a complex and multifaceted neuropeptide signaling system.

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